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# **Fluorescent peptides for imaging of fungal cells**

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## **Abstract**

Fungal infections, especially with the advent of antimicrobial resistance, represent a major burden to our society. As a result, there has been an increasing interest in the development of new probes that accelerate the study of fungi-related biological processes and facilitate novel clinical diagnostic and treatment strategies. Fluorescence-based reporters can provide dynamic information at the molecular level with high spatial resolution. However, conventional fluorescent probes for microbes often suffer from low specificity. In the last decade, numerous studies have been reported on the chemical design and application of fluorescent peptides for both *in vitro* and *in vivo* imaging of fungal cells. In this article, we review different strategies used in the preparation of fluorescent peptides for pathogenic fungi as well as some of their applications in medical imaging and in mode-of-action mechanistic studies.

## 1 Introduction

2 Fluorescence imaging has revolutionised our ability to study biological processes in cells and  
3 intact organisms with high spatial and temporal resolution [1-3]. In the last decades, multiple  
4 chemical approaches have been reported for the development of fluorescent probes that  
5 enable direct visualisation of biomolecules and cells. These encompass broad diversity-  
6 oriented fluorescent libraries to identify novel biomarkers of cell states [4] as well as reactive  
7 fluorophores for targeted conjugation to small molecules and drugs [5-7]. Major advantages  
8 of fluorescent probes include: 1) high sensitivity with typically low limits of detection [8-10], 2)  
9 compatibility for multiplexed assays [11, 12], and 3) high versatility in multiple assay formats,  
10 from mechanistic studies [13, 14] to high-throughput screenings [15] and *in vivo* imaging [16].

11  
12 Fungi are eukaryotic microorganisms with relative similarities to mammalian cells. Although  
13 most fungi are not pathogenic, several species can cause severe fungal infections in humans  
14 and plants. Globally, there are more than 1.5 billion people suffering from fungal infections,  
15 ranging from mild chronic superficial infections to life-threatening invasive infections caused  
16 by four main pathogenic fungi: *Candida*, *Aspergillus*, *Cryptococcus* and *Pneumocystis*.  
17 Furthermore, fungi are also a major cause of disease in crops, causing major environmental,  
18 social and economic burden. Currently there are only a few antifungal drugs used for treating  
19 such infections, and the resistance against them has been increasing at an alarming rate over  
20 the last years. The need for new strategies to combat antifungal resistance both in medicine  
21 and agriculture have prompted numerous studies to understand the mechanism of antifungal  
22 drugs as well as to develop new diagnostic tools with enhanced resolution and speed. To this  
23 end, fluorescence imaging has largely facilitated the development of novel diagnostics and  
24 treatments with improved selectivity for fungi over host cells and other pathogens [17-21].

25  
26 Whereas fluorescent probes can be rationally designed to target specific targets or even cell  
27 types [22, 23], they are often conjugated to other chemical structures that confer them with  
28 molecular recognition properties for enhanced target specificity. These can include small  
29 molecules [24], antibodies [25] as well as peptides, among others. Peptides are excellent  
30 scaffolds for the preparation of fluorescent molecular probes. In addition to low cost and well-  
31 established protocols for their parallel synthesis [26, 27], peptide conjugates can achieve high  
32 target specificity, including defined receptors and subtypes [28-31] as well as subcellular  
33 structures [32]. In this article, we will review some of the advances during the last decade in  
34 the preparation and evaluation of peptide-based imaging probes for the detection and  
35 characterisation of fungal cells, from *in vitro* cellular assays to *in vivo* imaging in animals and  
36 in humans.

## **1. Fluorescent peptides for optical imaging of pathogenic fungi**

Fungal cells display differential patterns of phospholipid and sterol composition in membranes when compared to mammalian cells (e.g., ergosterol instead of cholesterol) [33], yet there are still limited fluorescent contrast agents with high specificity for fungal cells [34]. Ideally, fluorescent probes for imaging fungal cells must exhibit: 1) strong binding to fungi-specific components, 2) specific uptake and retention, and 3) high chemical and photo-stability. Peptides have been increasingly recognised as promising scaffolds to generate agents for molecular imaging, partially due to their ability to form highly specific interactions with their respective targets [35-37]. This section summarises recent progress on the preparation and application of fluorescent peptides for optical imaging of fungal cells *in vitro* and *in vivo*.

### **1.1. Fluorescent peptides for live-cell imaging**

Most peptides do not contain functional groups that allow direct visualisation by live-cell fluorescence imaging. Therefore, reporters such as fluorophores are introduced to enhance the contrast between targeted cells and their background [38]. Fluorescent peptides are versatile agents for multiple applications, from live-cell imaging with subcellular resolution to *in vivo* detection of fungal infections in preclinical models. Fluorescent peptides and peptidomimetics for fungal cells have provided insights on the mode-of-action of antimicrobial peptides and have also allowed researchers to visualise biological processes associated to fungal growth in real time.

Fluorescent peptides for imaging of fungal cells benefit from the fact that fungi are larger than most microorganisms (e.g., bacteria). To date, several antifungal peptides have been labelled with fluorophores to perform live-cell imaging studies in various fungal strains. Rhodamine-labelled MtDef4 and Dylight550-labelled MtDef5 (both plant defensins) were employed for imaging cell entry and subcellular localisation in two ascomycete fungi: the model fungus *Neurospora crassa* and the pathogenic fungus *Fusarium graminearum*. These studies helped to gain insights into the mechanism by which defensins exert their antifungal activity [39-41]. Subsequently, Muñoz *et al.* reported that both plant defensins MtDef1 and MtDef4 and their cognate peptides exhibited different inhibitory effects in the germination and fusions of *N. crassa*. In order to examine the correlation between their antifungal activity and intracellular localisation, rhodamine-labelled fluorescent analogues of the cognate peptides MtDef4, GMA4-C and GMA4-L were prepared and tested to reveal that their different subcellular distributions were associated with variable levels of inhibitory activity [42].

Puig *et al.* described a concentration-dependent membrane permeabilisation mechanism for the antifungal peptide BP15 in the pathogenic fungus *Stemphylium vesicarium* [43]. Fluorescein-labelled BP15 was shown to interact with the cell periphery and later internalise

1 within the hyphae. The authors also demonstrated that BP15 could not permeabilise or enter  
2 conidial cells (i.e., reproductive spores), suggesting the potential use of BP15 for controlling  
3 fungal infection in plants and crops [44]. Maurya *et al.* described an interesting application of  
4 fluorescent peptides as mode-of-action exploratory probes to study resistance mechanisms in  
5 the human pathogenic fungus *Candida albicans* [45]. The authors prepared fluorescent  
6 analogues of the transmembrane peptide mimics (TMPMs) targeting the efflux pump Cdr1,  
7 which is one of the most predominant targets in azole-resistant *C. albicans* from clinical  
8 isolates. Fluorescein-labelled TMPMs were used to study their interaction with the  
9 transmembrane helices of Cdr1 protein and proved to block the efflux of fluorescent agents  
10 from the azole-resistant *C. albicans*. This study has opened new avenues in the development  
11 of TMPMs as Cdr1 inhibitors to overcome multidrug resistance in *C. albicans* infections. The  
12 same research group has also reported two other antifungal peptides (i.e., VS2 and VS3)  
13 which exhibit cytotoxic activity against various pathogenic species. Fluorescein-labelled VS2  
14 and VS3 were used to analyse the kinetics of permeabilisation and also the resulting  
15 intracellular accumulation of the peptides. Their results suggested that peptides could make  
16 the cell membrane permeable to other molecules that are unable to internalise on their own.  
17 This allowed both VS2 and VS3 to work synergistically with other antifungals such as  
18 fluconazole to exhibit an improved antifungal activity [46].

19  
20 PAF26 is one of the most studied short antifungal peptides. It was identified from a  
21 combinatorial positional scanning antimicrobial peptide library and it shows preferential activity  
22 toward filamentous fungi over other tested microorganisms [47] with minimal cytotoxicity in  
23 human red blood cells [48] and keratinocytes [49]. Muñoz *et al.* prepared fluorescent  
24 analogues of PAF26 by incorporation of carboxytetraaminomethylrhodamine (TAMRA) and  
25 fluorescein isothiocyanate (FITC) and used them for live-cell imaging in combination with  
26 additional dyes (i.e., FM-4-64 as a membrane and endocytosis-staining dye, cDFFDA as a  
27 vacuolar dye, and propidium iodide as a dead cell marker). The authors observed that  
28 fluorescently-labelled PAF26 derivatives exhibited concentration-dependent penetration and  
29 cytotoxicity in *N. crassa* [50]. At high concentrations, PAF26 was primarily translocated across  
30 the plasma membrane by passive diffusion and exerted its fungicidal effect via an energy-  
31 independent process. On the other hand, at low concentrations PAF26 exhibited a different  
32 mechanism. Upon interaction with the fungal cell envelope, the peptide showed a progressive  
33 uptake through endocytosis with accumulation in vacuoles and subsequent transport to the  
34 cytoplasm coinciding with cell death [50]. Similarly, the antifungal peptide histatin-5 was also  
35 reported to display a concentration-dependent mechanism on its fungicidal action in *C.*  
36 *albicans*. At low concentrations, histatin-5 was internalised via receptor-mediated endocytosis  
37 into cell vacuoles, whereas the direct translocation through the plasma membrane was

1 reported at higher concentrations [51, 52]. Although PAF26 and histatin-5 are both positively-  
2 charged peptides, their chemical structures are remarkably different. PAF26 is a 6-mer  
3 whereas histatin-5 is a 24-mer, thus it is noteworthy that they exhibit similar concentration-  
4 dependent mechanisms of internalisation and antifungal activity. In another study, Ordonez *et*  
5 *al.* performed real-time tracking of the internalisation of fluorescent analogues of the human  
6 cathelicidin peptide LL-37 and the chicken cathelicidin 2 (CATH-2) in *C. albicans*. The authors  
7 showed that both LL-37 and CATH-2 exhibited rapid strong membrane staining, however their  
8 internalisation was energy-independent, suggesting direct membrane permeabilisation as the  
9 main mechanism of action [53]. Unlike histatin-5 and PAF26, both LL-37 and CATH-2 showed  
10 lack of fungal specificity and rather general antimicrobial properties, with almost identical  
11 mode-of-action in different non-fungal microorganisms [54-56]. Furthermore, several other  
12 short antimicrobial cationic peptides that exhibit endocytosis-independent internalisation have  
13 been reported to be non-specific for fungal cells. These results indicate that the initial  
14 interactions between PAF26 and histatin-5 with the fungal cell envelope are critical for their  
15 fungal selectivity over other microorganisms and suggest that biomolecules in the fungal cell  
16 envelope might be potential targets. The fact that both PAF26 and histatin-5 need to be  
17 translocated to the cytosol to exert their fungicidal action also suggests that they may also  
18 have additional intracellular targets [52, 57].

19  
20 Apart from the membrane-targeting mechanism discussed above, Iida *et al.* reported a  
21 vacuolar-targeting mechanism for polymyxin B in *Saccharomyces cerevisiae*. Polymyxin B is  
22 known as a cationic cyclic peptide that inhibits Gram-negative bacteria by targeting  
23 lipopolysaccharide and disrupting the outer bacterial membrane [58, 59]. It is worth noting that,  
24 by conjugating an environmentally-sensitive fluorophore (i.e., dansyl), the authors could  
25 observe the accumulation of polymyxin B in the vacuoles of fungal cells lacking  
26 lipopolysaccharide, suggesting a different mode-of-action in fungal cells. [60]

27  
28 The studies mentioned above demonstrate the utility of fluorescent peptides for *in situ* analysis  
29 of antifungal activity, however the impact of the fluorophores on the properties of the peptides  
30 have not been extensively evaluated. Most studies employ commercially available  
31 fluorophores that are conjugated to the N- or C- terminal groups. This strategy may cause  
32 alterations in short peptide sequences and might compromise their biological activity. To  
33 address this issue, Zhao *et al.* recently conducted a systematic combinatorial study to analyse  
34 the influence of different fluorophores on the hexapeptide PAF26 by preparing 12 different  
35 fluorescent analogues and comparing their cellular uptake and distribution using confocal live-  
36 cell imaging [61]. PAF26 was employed as a representative antimicrobial peptide with both  
37 cationic and hydrophobic regions. In addition to widely used fluorophores (e.g., TAMRA,

FITC), another 10 fluorophores covering the whole UV-visible spectrum (i.e., from blue to near-infrared fluorescence) and with variable physicochemical properties were used for labelling PAF26. This selection included conventional fluorescent scaffolds (e.g., cyanine, BODIPY), environmentally-sensitive dyes (e.g., NBD, dansyl, Nile Blue), pH-sensitive dyes (e.g., naphthalimide) and esterase-activatable fluorophores (e.g., fluorescein diacetate). By careful evaluation of the library, the authors concluded that the incorporation of different fluorophores into the sequence of PAF26 significantly influenced its intracellular localisation. Positively-charged fluorophores (e.g., styryl) favoured the interaction with anionic membranes, whereas negatively-charged fluorophores (e.g., FITC) preferentially accumulated in intracellular vacuoles. Near-infrared fluorophores (e.g., cyanine) provided bright cytosolic staining and good discrimination from background signals, both *in vitro* and using whole-body *in vivo* fluorescence imaging. Additionally, the pH-sensitive fluorophores naphthalimide facilitated the visualisation of vacuolar acidification in fungal cells. These results suggest that peptides including bespoke fluorophores may represent a good strategy to visualise specific intracellular processes in fungal cells with subcellular resolution.

## 1.2. Fluorescent peptides for *ex vivo* tissue and *in vivo* imaging

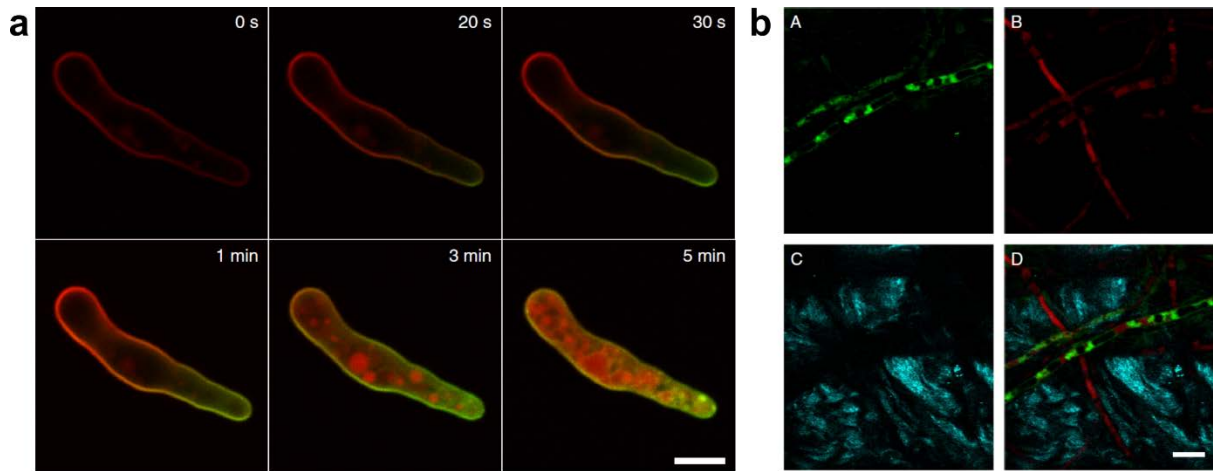
Over the past decades, medical imaging technologies have emerged as powerful tools for early-stage diagnosis and non-invasive monitoring of disease progression, including patient stratification and response to therapy [62, 63]. Peptide-based optical probes have been increasingly applied for different pathological conditions, including cancer and bacterial infections, largely due to their good target specificity, marginal cytotoxicity and low immunogenicity [64]. However, imaging fungal infection still remains largely unexplored in the medical arena, with only a few successful examples of peptide-based imaging probes.

*C. albicans* is a common pathogenic fungus in humans that can cause skin, oral gastrointestinal and urogenital infections as well as fatal systemic infections in immunocompromised patients [65, 66]. For the infections to grow, *C. albicans* need to first aggregate and adhere to host cells. Of note, the biofilms formed by the fungal cells increase the resistance against conventional antimicrobial treatments [67, 68]. The *C. albicans* agglutinin-like sequence (Als) form functional amyloid patches on the surface of host cells and plays an important role in cell adhesion and biofilm formation during the development of the infection [69-71]. Garcia *et al.* reported the development of a fluorescent amyloid-forming peptide Als-5p able to bind *C. albicans* in both yeast and hyphal forms. The authors also demonstrated that the fluorescent peptide Als-5p could bind to fungi in gastrointestinal tissue samples from candidiasis patients [72]. Compared to traditional amyloid-binding dyes, such



1 as thioflavin T, the fluorescent Als-5p peptide probe showed good selectivity for amyloids of  
2 fungal origin.

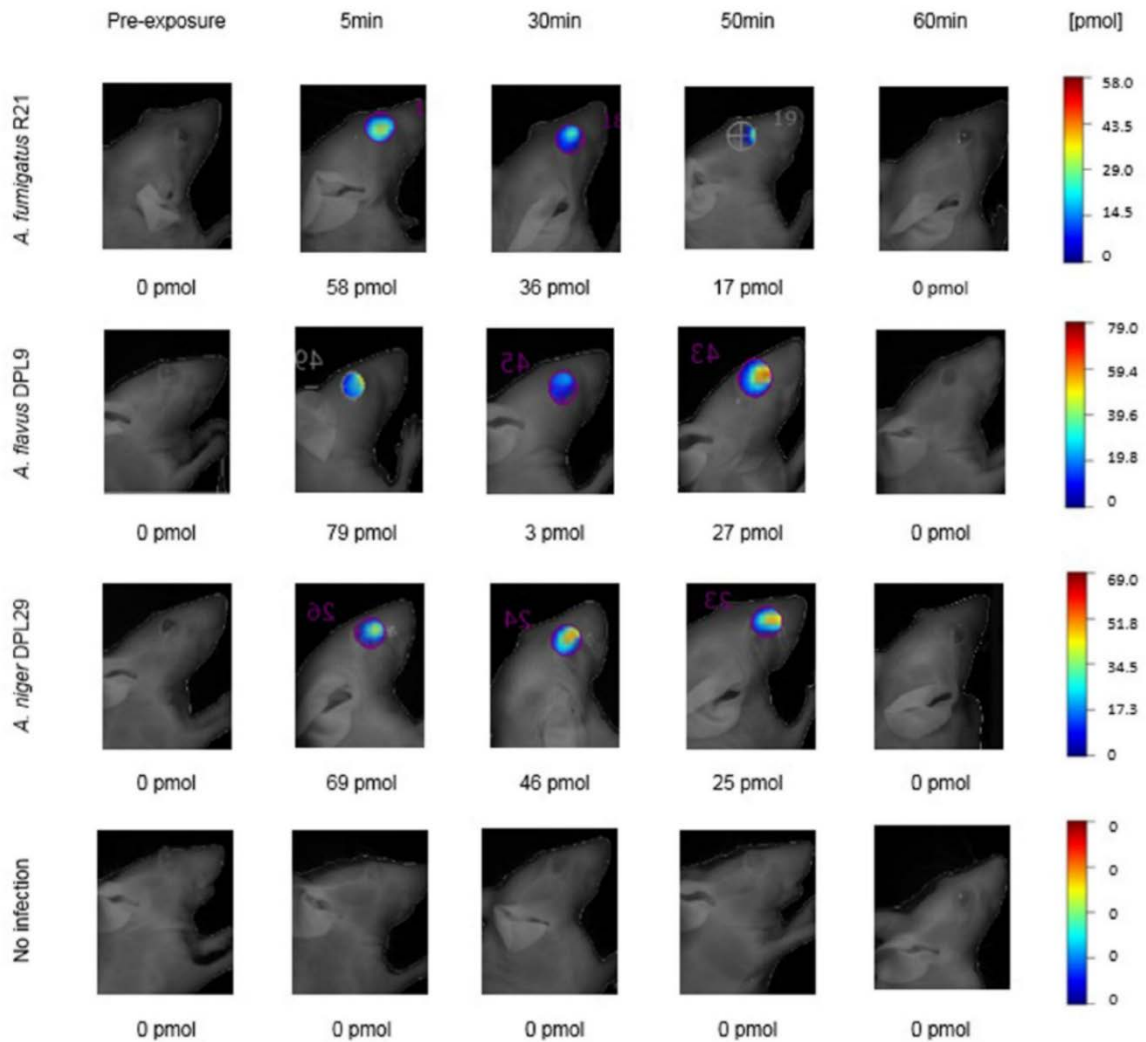
3  
4 Invasive pulmonary aspergillosis (IPA) is a fatal infection caused by *Aspergillus* species in  
5 immunocompromised patients. Current diagnostic approaches rely on cultures from  
6 bronchoalveolar lavage fluid and peripheral blood, which are time-consuming and lead to  
7 mortality rates of IPA patients over 40% due to inefficient diagnostic tools [73]. Mendive-Tapia  
8 *et al.* described a novel peptide-based imaging probe with selective binding to *A. fumigatus* in  
9 *ex vivo* human lung tissue [32, 74] (**Figure 1**). Notably, the authors developed a peptide with  
10 a fluorogenic core for real-time imaging of fungal species *in situ*, without the need of additional  
11 washing steps. In other studies, Morisse *et al.* and Akram *et al.* conducted assays using  
12 fluorescent peptides in fiber-based confocal fluorescence endomicroscopy (FCFM) [75, 76].  
13 Pioneered by Thiberville and co-workers, FCFM allows *in vivo* imaging of lung microstructures  
14 during bronchoscopy [77]. Morisse *et al.* employed a fluorescein-labelled cyclic peptide while  
15 Akram *et al.* employed an NBD-labelled peptide, both showing good selectivity for fungi over  
16 host cells. All these studies provide innovative strategies for the future development of  
17 improved IPA diagnostic tools.



**Figure 1. Fluorescence images of *A. fumigatus* in vitro and in ex vivo human tissue.** a) High-resolution fluorescence confocal images of the fungal pathogen *A. fumigatus* after incubation with a cell membrane counterstain (red) and following addition of the fluorescent peptide 8 (2  $\mu$ M, green). Peptide 8 initially interacts rapidly with the apical plasma membrane of *A. fumigatus*, and its staining then shifts toward the base of the germling cell within a few minutes.  $\lambda_{exc}$ : 496 nm,  $\lambda_{em}$ : 505–550 nm (green);  $\lambda_{exc}$ : 570 nm,  $\lambda_{em}$ : 585–650 nm (red). Scale bar: 5  $\mu$ m. b) Multi-photon fluorescence microscopy of ex vivo human pulmonary tissue after incubation with RFP-expressing *A. fumigatus* and peptide 8. Fluorescence images of peptide 8 (5  $\mu$ M) (A), RFP-expressing *A. fumigatus* (B), second harmonic generation from collagen fibres (C) and merged (D) in ex vivo human lung tissue. Scale bar: 10  $\mu$ m. Images reproduced with permission from references 32 (a) and 74 (b).

Fungal keratitis (FK) is a major cause of ocular morbidity and blindness. It globally affects contact lens wearers and patients experiencing trauma in the eye. FK infections are predominantly caused by two fungal genera, *Aspergillus* and *Fusarium*. Around 50% of FK patients lose sight in the infected eye and over 13,000 eyes are lost completely per year in worldwide [78, 79]. Early diagnosis of FK is critical to ensure the best possible therapeutic outcome. However, current diagnosis relies on direct *in vivo* confocal microscopy (IVCM) and microbiological culture of corneal scrapings [80]. IVCM requires expensive equipment and is mostly limited to well-resourced hospitals. Microbiological cultures of corneal scrapings reveal infections in 40-85% of cases, but they take weeks and can miss deep infections. Therefore, the development of safe, rapid, and sensitive diagnostic probes is necessary to improve early-stage FK diagnosis. Lee *et al.* recently reported the lipopeptide caspofungin coupled to near-infrared fluorophore DDAO (CSF-DDAO) as a tomographic imaging probe for rapid FK diagnosis [81]. The authors demonstrated that CSF-DDAO efficiently labelled various *Aspergillus* species *in vitro*. They also used a liposome-encapsulated CSF-DDAO for the detection of various species including *A. fumigatus*, *A. niger* and *A. flavus* in a mouse model

of FK within 10 min of post topical application (**Figure 2**). This study constitutes an excellent example of the potential of fluorescent peptides for enhanced diagnosis of fungal infections *in vivo*.



**Figure 2. Fluorescence imaging of fungal keratitis in mice.** SKH1 mice infected intrastromally with *A. fumigatus*, *A. flavus* or *A. niger* were tomographically imaged 24 h post-infection at the indicated time points (i.e., pre-exposure, 5, 30, 50, and 60 min post-exposure) with the CSF-DDAO probe using the 680 nm fluorescence emission filter. Fluorescence-based quantification for the probe (in picomoles) is shown for every image and depicted on the intensity map. Images reproduced with permission from reference 81.

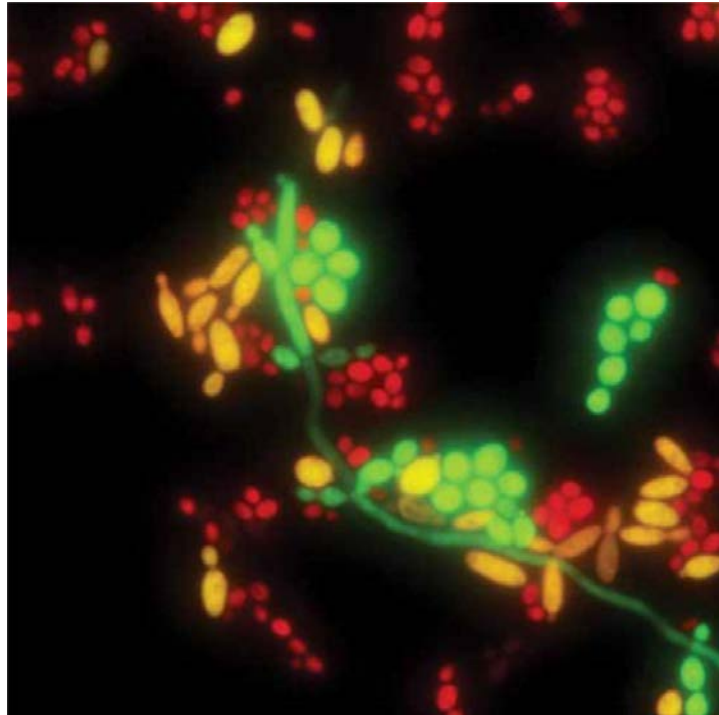
## 2. Fluorescent peptide nucleic acids for *in situ* hybridisation

Fluorescence *in situ* hybridisation based on peptide nucleic acids (PNA-FISH) is a commercial Food and Drug administration (FDA)-approved assay for the detection and identification of invasive microbial infections [82]. This method is based on fluorescently-labelled PNA probes that recognise and bind to complementary ribosomal RNA regions of microorganisms for direct identification at a single-cell level. Notably, because these agents show high susceptibility to small changes on target RNA sequences, they are also able to discriminate between closely-related pathogens. Several PNA-FISH assays targeting either 18S, 26S or 28S rRNA regions of *Candida* species have been described [83, 84]. PNA probes are synthetic DNA mimics comprising a hydrophobic and uncharged polyamide-type backbone to which individual nucleobases are attached. Thus, the hydrophobic nature of the probe facilitates permeability -although methanol fixation is often used- and the lack of electrostatic repulsion leads to stronger binding to the target sequence and faster hybridisation kinetics. Overall, the probes show improved resistance to nucleases and proteases as well as higher specificity and affinity than DNA probes.

PNA-FISH allows rapid identification of the infectious agents at both genus and species level directly from peripheral blood samples (**Figure 3**). Importantly, this assay provides high sensitivity and specificity with no requirement of subcultures as in conventional blood culture methods [85]. The first generation of PNA-FISH agents were used to detect and differentiate the five most common *Candida* species (sensitivity 97.5-98.9%, specificity 98.2-100%) in three main groups according to the wavelength of the fluorescence emission: *C. albicans*/*C. parapsilosis* (green fluorescence), *C. tropicalis* (yellow fluorescence) and *C. glabrata*/*C. krusei* (red fluorescence) [86]. The identification of multiple *Candida* species by differential fluorescence emission also provided valuable information of the resistance of the strains to antifungal treatments. Thus, whereas *C. albicans* and *C. parapsilosis* were sensitive to commonly prescribed antifungals (i.e., fluconazole, echinocandin), *C. tropicalis*, *C. glabrata* and *C. krusei* were more prone to become resistant to these treatments. The second generation of PNA-FISH probes significantly reduced the time needed for probe incubation (i.e., from 90 to 30 min), further shortening the time-to-result outcome. This second generation was able to distinguish the common species *C. albicans* (green fluorescence), *C. parapsilosis* (yellow fluorescence) and *C. glabrata* (red fluorescence) with sensitivities and specificities at around 99.7 and 98%, respectively. The high sensitivity and specificity of this PNA-FISH assay was validated in a multi-institutional study, where 244 clinical blood samples were evaluated for the selective detection of *C. albicans* [87]. In another study, *C. albicans* and *C. Glabrata* were identified alone or in combination with other pathogens in significantly shorter times [88]. The detection of fungal pathogens by PNA-FISH typically comprises four main steps: 1) fixation of the sample to prevent loss of cell integrity, 2) hybridisation of the fluorescent probe

1 to the target sequence at a specific temperature (40-60°C range), 3) washing to remove  
2 unhybridised PNA probe, and 4) detection of the probe by fluorescence microscopy. Critically,  
3 the experimental conditions during the hybridisation step (e.g., concentration, temperature,  
4 pH, salt concentration, etc.) must be optimised to ensure hybridisation and a high degree of  
5 standardisation. In an optimisation study of the 28S rRNA targeting probe (P-Ca726) for the  
6 identification of *C. albicans*, Kim *et al.* showed that the use of the denaturant formamide  
7 reduced non-specific binding and increased overall performance [89]. An important feature of  
8 PNA-FISH assays is the discrimination of *C. albicans* from closely related atypical species,  
9 avoiding misidentification of pathogens in blood patient samples. Oliveira and co-workers  
10 showed that *C. albicans* and *C. dubliniensis* PNA-FISH probes could be simultaneously used  
11 to identify both *Candida* species without cross-hybridisation [90]. Nevertheless, some cross-  
12 reactivity with other non-clinically relevant *Candida* species has been reported in other studies  
13 (e.g., *C. lambica*, *C. Rhodotorula mucilaginosa*) [91]. The appropriate identification of *Candida*  
14 species is crucial to provide adequate treatment and reduce times and associated costs  
15 (**Figure 3**). A comparative study of PNA-FISH and routine methods in hospitalised patients  
16 with candidemia proved that the times needed to prescribe the correct therapy and the  
17 associated costs were significantly reduced after implementation of the PNA-FISH assay, with  
18 savings of over \$400 per patient [92]. In another study, an evaluation of the clinical impact of  
19 a second-generation assay (i.e., PNA FISH Yeast Traffic Light assay) concluded that, after  
20 the analysis of 54 patient isolates, the implantation of the test would have led to changes in  
21 antifungal therapy in more than 29% of the cases if the results of the PNA-FISH assays had  
22 been available [93].

23 The detection and characterisation of other fungi by PNA-FISH has also been described.  
24 Teertstra *et al.* reported the visualisation of *Schizophyllum commune* and *Aspergillus niger*  
25 colonies, showing the first report of PNA probes for *in situ* hybridisation of mRNA in  
26 filamentous fungi [94]. Subsequently, Nakada *et al.* reported the first localisation study of  
27 *Phanerochaete chrysosporium* and *Postia placenta* fungi in wood by FISH [95]. PNA-FISH  
28 also represents a promising strategy for the diagnosis of *A. fumigatus* as most current methods  
29 only allow the identification of the fungus at the genus level [96]. Finally, despite its excellent  
30 sensitivity, specificity and speed, some limitations of PNA-FISH assays must be taken into  
31 consideration: 1) assays are expensive and require experienced and well-trained individuals  
32 for correct identification of the fungi, 2) pathogen concentrations over 10<sup>5</sup> colony-forming units  
33 mL<sup>-1</sup> are needed for adequate detection, and 3) PNA-FISH is a target-oriented assay that  
34 requires an estimation of the possible pathogens that are expected to be detected.



**Figure 3. Fluorescence identification of multiple fungal species using AdvanDx PNA-FISH.** Multiplex fluorescence image of the five most common *Candida* species: *C. albicans*/*C. parapsilosis* (green fluorescence), *C. tropicalis* (yellow fluorescence) and *C. glabrata*/*C. krusei* (red fluorescence). Image reprinted with permission from OpGen, the manufacturer of the test.

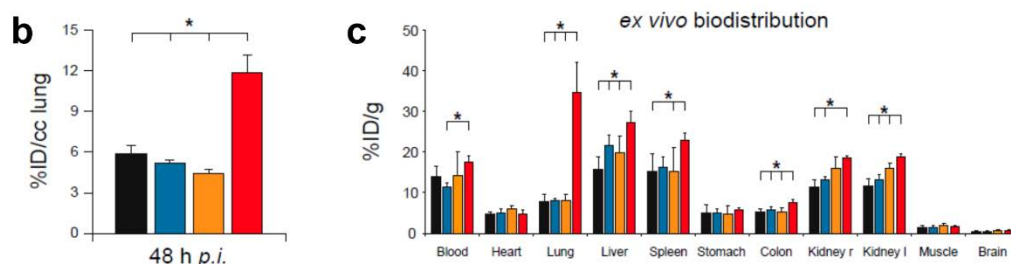
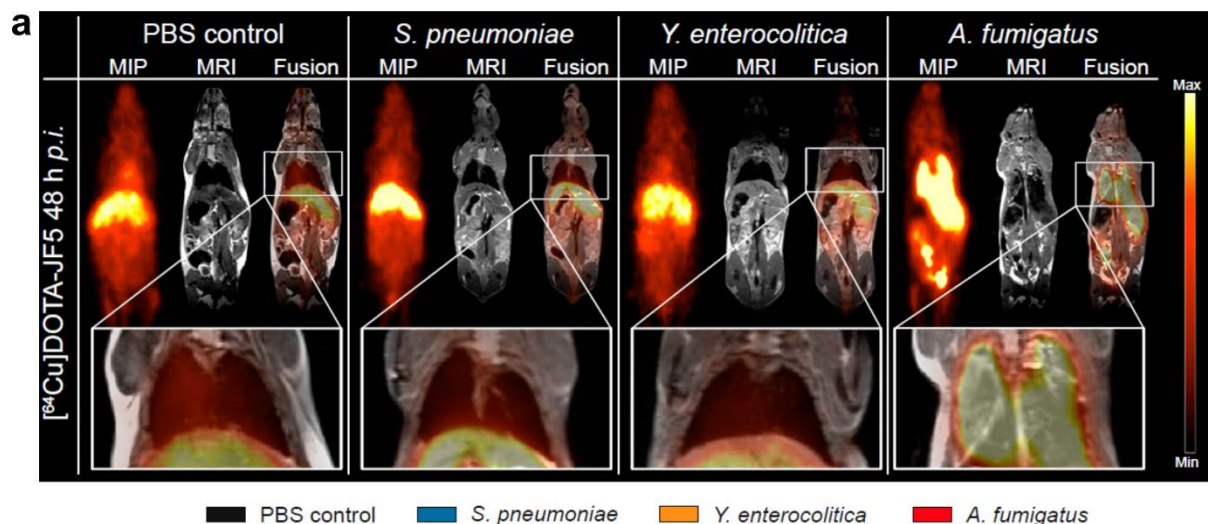
### 3. Alternative modalities for non-optical *in vivo* imaging

Although the direct visualisation of cells and tissues using light has been one of the most informative approaches in diagnostic medical imaging, its limited depth of penetration (less than 1 cm) has hampered its widespread application for *in vivo* clinical imaging studies. This can partially overcome with the use of near-infrared dyes (i.e., emitting in the spectral range from 650 nm to 900 nm) [97, 98], which provide deeper tissue penetration and can be used for whole-body fluorescence tomography in preclinical models.

Non-optical imaging modalities with limitless penetration characteristics, such as positron emission tomography (PET), magnetic resonance imaging (MRI) and computed tomography (CT), are alternative technologies with broad use in clinical imaging [99]. In the clinic, the most common reagent used for PET imaging is the  $^{18}\text{F}$ -labeled imaging agent [ $^{18}\text{F}$ ]-2-fluoro-2-deoxy-glucose (FDG). FDG was first synthesized by Ido *et al.* in 1978 [100] and it is an analogue of glucose whereby the  $\text{C}_2$  hydroxyl group is replaced by the radioactive fluorine atom  $^{18}\text{F}$ . The accumulation of FDG reflects the metabolic requirements of cells, and as such, it has been used to detect cancer cells, which present metabolic demands that are different from most normal cells [101]. Recently FDG has been also used in PET/CT imaging for detecting and guiding management of invasive fungal infections, with improved performance

1 over CT alone for the localisation of infection, dissemination and response to therapy in human  
2 patients [102].

3 The limited specificity of FDG has, however, prompted the development of reagents with  
4 higher selectivity for fungal cells in order to reduce potential off-target signals. In this context,  
5 two recent studies have demonstrated the translational potential of PET contrast agents for  
6 imaging fungi in preclinical models and in humans. Rolle *et al.* developed a novel probe for  
7 non-invasive detection of *A. fumigatus* infection in the lungs of mice using PET and MRI [103]  
8 **(Figure 4)**. The authors generated a  $^{64}\text{Cu}$ -DOTA-labeled monoclonal antibody specific to *A.*  
9 *fumigatus* that allowed specific localisation of fungi in infected lungs. Notably, this new PET  
10 tracer ( $^{64}\text{Cu}$ -DOTA-JF5) was able to distinguish fungal from bacterial lung infections and,  
11 unlike FDG, discriminated Aspergillosis from generic increases in metabolic activity  
12 associated with lung inflammation. Peptides, which are smaller constructs than antibodies,  
13 typically offer improved biodistribution for *in vivo* studies. This has been recently proven by  
14 Ebenhan *et al.* with a first in-human study of  $^{68}\text{Ga}$ -NOTA functionalised ubiquicidin (UBI<sub>29-41</sub>),  
15 a cationic-rich peptide with high affinity for fungal cells [104]. Initial assays assessed the  
16 performance of the tracer *in vitro* using different bacterial and fungal strains, followed by  
17 PET/CT-imaging biodistribution and pharmacokinetics studies on non-human primates.  
18 Finally,  $^{68}\text{Ga}$ -NOTA-ubiquicidin was tested in a small cohort of humans and proven as nontoxic  
19 radiopharmaceutical with huge potential for the translational imaging of fungal infections in the  
20 clinic.



**Figure 4. A disease-specific PET-tracer for the detection of *A. fumigatus* lung infection.**

a) Sagittal maximum intensity projections of MRI and fused PET/MRI images of PBS-treated mice and *S. pneumoniae*-, *Y. enterocolitica*- and *A. fumigatus*-infected mice injected with  $^{64}\text{Cu}$ -DOTA-JF5 (48 h after infection). Tracer injection demonstrates highly specific accumulation in *A. fumigatus*-infected lung tissue compared to bacterial-infected or sham-treated animals.

b) Quantification of *in vivo* PET images with significantly higher uptake of  $^{64}\text{Cu}$ -DOTA-JF5 in the lungs of *A. fumigatus*-infected animals compared to the lungs of control animals.

c) *Ex vivo* biodistribution show significantly higher uptake of  $^{64}\text{Cu}$ -DOTA-JF5 in the lungs of *A. fumigatus*-infected animals compared to the lungs of control animals. Black bars, PBS-treated controls; blue bars, *S. pneumoniae*-infected mice; orange bars, *Y. enterocolitica*-infected mice; red bars, *A. fumigatus*-infected mice. Reproduced from reference 103.



**Table 1.** Summary table of peptides discussed in this article.

Peptide	Sequence	References
<b>MtDef1</b>	RTCENLADKYRGPCFSGCDTHCTTKENAVSGRCRD	39, 42
	DFRCWCTKRC	
<b>MtDef4</b>	RTCESQSHKFKGPCASDHNCASVCQTERFSGGRCR	40, 41
	GFRRRCFCTTHC	
<b>MtDef5</b>	MTSSASKFYTIFIVCLAFLFISTSEVEAKLCQKRSTT	41
	WSGPCLNTGNCKRQCINVEHATFGACHRQGGFGFAC	
	FCYKKC	
	APKKVEPKLCERRSKTWSGPCLISGNCKRQCINVEH	
	ATSGACHRQGIGFACFCKKKC	
<b>GMA4-C</b>	GRCRGFRRRCFCTTHC	42
<b>GMA4-L</b>	RGFRRR	42
<b>BP15</b>	KKLFKKILKVL	43,44
<b>VS2</b>	KWFWKFWK	45
<b>VS3</b>	KWFWKFWKFVK	45
<b>PAF26</b>	RKKWFW	50-52
<b>Peptide 8</b>	c[RKKW(BODIPY)FWG]	74
<b>Histatin-5</b>	DSHAKRHHGYKRKFHEKHSHRGY	50-52
<b>UBI<sub>29-41</sub></b>	TGRAKRRMQYNRR	104
<b>LL-37</b>	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	53-56
<b>CATH-2</b>	RFGRFLRKIRRFPRKVTITIQGSARF	53-56
<b>Als-5p</b>	SNGIVIVATTRTV	72

## Conclusions

The high sensitivity, multiplexing capabilities and low cost of fluorescence-based analytical techniques have favoured the emergence of optical imaging as a complementary tool to well-established clinical imaging modalities, such as MRI, PET and CT. A key aspect in the application of optical imaging involves the preparation of highly specific molecular probes for direct visualisation of fungal pathogens under live-cell conditions. To this end, fluorescent peptides have emerged as powerful scaffolds for both mechanism-oriented biological studies as well as clinical use. Peptide-based probes are highly versatile and amenable to combinatorial chemistry, offering the possibility to incorporate variable fluorescent labels at will to suit different applications. Peptides also exhibit low immunogenicity and generally good biocompatibility with cells and tissues, being ideal for non-invasive mechanistic cell-based assays as well as *in vivo* imaging studies. With our improved understanding of protein-protein interactions, peptide chemists will be able to design novel peptide probes as enhanced diagnostic tools and therapeutics for direct patient benefit.

## Abbreviations

TMPMs: transmembrane peptide mimics

TAMRA: carboxytetraaminomethylrhodamine

FITC: fluorescein isothiocyanate

cDFFDA: Oregon Green 488 carboxylic acid diacetate

CATH-2: cathelicidin 2

BODIPY: borondipyrromethene

NBD: nitrobenzoxadiazole

Als: agglutinin-like sequence

IPA: invasive pulmonary Aspergillosis

FCFM: fiber-based confocal fluorescence endomicroscopy

FK: fungal keratitis

IVCM: *in vivo* confocal microscopy

CSF-DDAO: Caspofungin-7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One)

PNA-FISH: fluorescence *in situ* hybridization using peptide nucleic acids

PET: positron emission tomography

CT: computed tomography

MRI: magnetic resonance imaging

FDG: <sup>18</sup>F- 2-deoxyglucose

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7   65<sup>th</sup> birthday for his outstanding contributions to the field of peptide chemistry.

8  
9   **Competing financial interests**

10   The authors declare no competing financial interests.

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